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PRINCIPAL INVESTIGATOR: James D. Morre

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West Lafayette, Indiana 47907

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West LaFayett, Indiana 47907

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This project has as its basis the discovery in my laboratory of a unique growth-related protein at the external cell surface. The protein, designated tNOX, is an antitumor drug-responsive hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity. In metastasis models, tNOX inhibitors prevent the invasive spread of cancer. The present project was to develop one or more lead drugs to prevent metastases in a breast cancer model. Glaucarubolone, a natural product tNOX inhibitor, both free and conjugated to polyethylene-glycol 5000, was prepared in quantities sufficient for animal testing. The EC_{50} for the glaucarubolone on the growth of 4T1 mouse mammary carcinoma cells was determined to be > 1 μM. That for the conjugate was < 1 μM. Free glaucarubolone supplied intra-tumorally to mice bearing transplanted mammary carcinomas was toxic and ineffective in the prevention of the formation of lung metastases. The conjugate was not toxic and a response was seen at the highest concentration tested of 5 mg/injection/mouse. In a collagen invasion assay, the glaucarubolone-PEG conjugate was effective at concentrations between 10^{-8} and 10^{-10} M. The conjugate will be redesigned to improve solubility and intratumor distribution to facilitate attainment of therapeutic doses.

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INTRODUCTION

My laboratory has discovered and characterized a 34 kD protein associated with the cell surface of cancer cells. The protein exhibits protein disulfide-thiol interchange (TIP) and NADH oxidase (NOX) activities that are inhibited by a small cadre of quinone site inhibitors. The site is at the external cell surface and drugs need not enter cells to be effective Using a B-16 mouse melanoma model, we have demonstrated that inhibition of the TIP and/or NOX activities of the melanoma cell plasma membrane results in blockage of the metastatic spread of the cancer. As metastatic spread is the principal cause of treatment failure in breast cancer, this proposal is to extend these findings to metastatic breast disease. Vanilloids, together with quassinoids having demonstrated activity against breast cancer, were to be immobilized to impermeant supports to increase their margin of safety and to be tested as anti-metastatic agents of potentially high specificity and selectivity for use in breast cancer management.

The purpose of the study proposed is to extend findings of anti-metastatic activity of vanilloid and quassinoid antitumor agents, free and conjugated to impermeant supports, to a murine metastatic breast cancer model. The findings are to provide proof-of-concept of the potential utility of these drugs targeted to a cancer-specific cell surface NADH oxidase as drug leads for development of new clinical agents in the management of metastatic breast disease.

The hypothesis under investigation is that the drug-responsive cell surface protein with NADH oxidase activity specific to cancer cells is critical to the metastatic process. Expression of the activity would provide metastatic potential. Inhibition of the expressed activity would prevent metastasis. The normal function of the drug-responsive NADH oxidase protein is that of a protein disulfide-thiol interchange protein involved in physical membrane displacement critical to cell enlargement following cell division and to invasion during metastatic spread. The unregulated and drug-responsive form at the cancer cell surface may correlate with metastatic potential.

The growth factor- and hormone-responsive protein disulfide-thiol interchange protein of the plasma membrane with NADH oxidase activity (1,2) appears to be involved functionally in physical membrane displacements related to cell enlargement and cancer metastasis (1). In transformed cells, the activity is constitutively activated and no longer hormone- and growth factor-responsive (3,4). Additionally, the cancer form of the activity (designated tNOX) is specifically inhibited by thiol reagents (5) and by a small cadre of antitumor drugs whose sites of action, until now, have remained elusive. These drugs include the antitumor sulfonylureas (6), the antitumor quassinoids (7) and certain vanilloids such as capsaicin (8-methyl-N-vanillyl-6-noneamide) (4). Drugs of all three groups appear to occupy a quinone site on the protein, a site that also can be occupied in a functional manner by the natural quinone cofactor, ubiquinone (coenzyme Q_{10}) (8).

The protein is anchored via its C-terminus in the outer leaflet of the plasma membrane. Both protein and drug site are external and drugs directed to the responsive cell surface NADH oxidase need not enter the cells to be effective.

HeLa and other cancer cells inhibited by drugs targeted to the responsive NADH oxidase fail to enlarge normally following cytokinesis (9). Cells must reach a minimal size in order to re-enter mitosis (10). The small cells resulting from treatment with antitumor quassinoids, vanilloids or sulfonylureas, subsequently fail to enter mitosis and undergo apoptotic cell death.

The basis for prevention of cell enlargement by tNOX inhibitors has been explained on the basis that the normal function of the enzymatic activity measured as an NADH oxidase may be to catalyze a protein disulfide-thiol interchange involved in physical membrane displacements important to growth and especially cell enlargement. In cancer, these reactions become unregulated and the unregulated activity becomes responsive to the small cadre of potential antitumor drugs listed above. When inhibited, the cell surface displacement activities associated with growth and invasion of cancer cells are blocked.

Preliminary studies were with human mammary adenocarcinoma (4) and human lines in culture. Using the B-16 mouse melanoma carried in C57BL/6 mice, experiments were extended to animals for the vanilloids and with a transplantable murine adenocarcinoma (17/A/Adr) for the quassinoids. Growth of both was effectively reduced. An unexpected outcome of these studies, however, was the effectiveness of the vanilloids to completely prevent metastatic spread of the B-16 to melanoma line with both capsaicin and vanillylamine.

Also effective as a potent NOX inhibitor was the antitumor quassinoid, glaucarubolone. Glaucarubolone is a representative of a series of both naturally occurring and chemically-modified plant products from the family Simbouriaceae with potent anti-cancer activity (7). These compounds have been tested extensively on M17 Adr mouse mammary carcinoma cells. Especially effective was the hydroxy analog of glaucarubolone which demonstrated solid tumor selectivity to both murine and human cells but also selectivity to M17/adr. Additional specificity to glaucarubolone has been imparted by conjugates involving the C-15 hydroxyl. Effectiveness of a C-15 acylated analog ($R=COCH_2NMe_2$) of glaucarubolone against MAM 16/C/RP cells) (% T/C of 16) has been demonstrated. A 2% T/C equal to < 10 constitutes a highly active agent by NCI standards and would represent > 1 log cell kill. Since the drug-responsive site of the NADH oxidase is located at the cell's exterior, inhibitory drugs directed to the site need not enter cells to be effective. Impermeant conjugates, therefore can be used an antitumor agents. These conjugates also help overcome a potential drawback of the quassinoid antitumor agents, that of a relatively high inherent cytotoxicity and narrow therapeutic ratio. By reducing or eliminating unspecific cytotoxicity and restricting the drug to the cell surface target, conjugation of quassinoids to

impermeant supports greatly increases efficacy and reduces toxicity with a corresponding 10- to 100-fold potential increase in the margin of safety.

One such conjugate where preliminary data are available is a conjugate of glaucarubolone through the C-15 hydroxyl to amino polyethylene glycol (PEG). The amino-PEG-glaucarubolone is more effective than free glaucarubolone in inhibiting the target NADH oxidase of plasma membrane, exhibits enhanced water solubility compared to glaucarubolone and is more effective than glaucarubolone in inhibiting the growth of HeLa cells (Addenda).

This project is to extend findings with cell-surface NADH oxidase inhibitors from the B-16 mouse melanoma metastatic model to a mouse mammary adenocarcinoma model with a view toward establishment of proof-of-concept for the use of immobilized inhibitors of cell surface NADH oxidase as anti-metastatic agents in the management of breast disease.

BODY

EXPERIMENTAL METHODS AND PROCEDURES

Female BALB/c mice 8 to 12 weeks old were obtained from a commercial supplier (Harlan Industries, Indianapolis, IN) and housed in the Purdue University Small Animal Facility following good animal practice procedures adopted by the University-wide animal care committee.

Tumor cell lines - Tumor subpopulation line 4T1 arising from a BALB/cf C3H mouse were utilized (16). The cells were grown in DME-10, Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). To prepare cells for injection, flasks were rinsed with 0.25% trypsin in 0.5% EDTA and washed once, and suspended in DME-10.

Experimental metastasis - Cells from monolayer culture were suspended in Hank's buffered salt solution and 1 x 10^5 cells were injected s.c. into mice in the subscapular region in a volume of 0.1 ml. Primary tumors were measured twice a week in 2 perpendicular dimensions using a vernier caliper. Tumor mass in cm^3 was calculated by the formula a x $b^2/2$, where b is the smaller and a is the larger of the two dimensions. To estimate doubling time, the values were fitted to an exponential growth curve using linear regression analysis of the logarithm of tumor volume.

Anti-metastatic drugs were administered intratumoral on alternate days beginning after palpable tumor masses were discernible. Appropriate solvent and sham-injected controls were included. At 15 days post tumor implantation mice

were sacrificed and major organs (e.g. lung, liver, lymph nodes) were examined for evidence of metastases.

Invasion *in vitro* - Embryonic chick heart fragments were incubated with adenocarcinoma cells (18) . After 4 days of confronting culture in liquid medium, the heart fragments were fixed in Bouin solution, processed for paraffin embedding and sectioned serially. Consecutive sections, stained with hematoxylin-eosin were used for histological evaluation of the interaction between the heart fragments and adenocarcinoma cells. The extent of interactions were assigned a numerical rating as described (19). To examine the effect of potential antimetastatic agents on the interaction between adenocarcinoma cells and the heart fragments, the agents were dissolved in the medium during the preculture period (4 days) of the heart fragments, during the confronting culture period or during both periods. Concentrations ranged between 0.000 and 0.100 mM. Reversibility of a possible anti-invasive effect of the agents was tested by changing the medium after 4 days of confronting culture for agent-free medium and further culturing during another 4 days.

Viability of aggregates and heart fragments - Individual heart fragments and adenocarcinoma aggregates were treated with different concentrations of potential antimetastatic agents for 8 days and 4 days respectively, under similar circumstances as for confronting cultures. The aggregates were then explanted in agent-free culture medium on a tissue culture plastic substrate as described (19). The number of explants showing radial outgrowth on the substrate versus the total number of explants were considered as a measure of cytotoxicity.

Growth of cell aggregates - Spheroidal adenocarcinoma aggregates with an initial diameter of 0.2 mm were grown in suspension culture for 7 days in the presence or absence of the antimetastatic agents under test. Changes in volume were calculated from daily measurements of aggregate diameters using an inverted microscope fitted with an ocular micrometer.

Colony formation - Approximately 1 ml portions of solid agar medium containing 1.5×10^4 adenocarcinoma cells in suspension were incubated in 35 mm diameter plastic Petri dishes at 37°C for 7 days. Culture dishes were then placed individually on a cross-hatched plastic matrix. Groups of cells (> 3 cells) were counted and colony diameters measured.

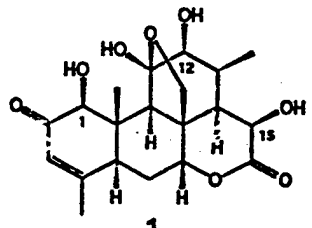
Morphology and motility of individual adenocarcinoma cells - Observations were made on HeLa cells in 25 cm^2 plastic tissue culture flasks in medium with or without potential antimetastatic agents. For observation and image analysis, video films were made using a Wild inverted microscope with phase contrast optics (10 x objective) in a thermostatic chamber (37 ± 0.5°C), a National Panasonic WV-1850C video camera and a Sony Umatic VO-585P video recorder equipped with an AC-580 animation control unit and a time-date generator. Analysis of morphology

was carried out on real-time video films, and analysis of motility was on time-lapse video films using a Bueckler microscaler.

Isolation of mouse mammary plasma membrane – Procedures followed those developed in our laboratory for isolation of plasma membranes from human mammary adenocarcinoma and other cultured cell lines using aqueous two-phase partition (4). The purity of the plasma membrane was determined by electron microscope morphometry and assay of marker enzymes.

Spectrophotometric measurement of NADH oxidase - NADH oxidase activity was determined at 37°C as the disappearance of NADH measured at 340 nm. Activity was measured using a Hitachi U3210 with stirring and continuous recording over 5 min intervals. The reaction mixture contained 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN and 150 μM NADH. A millimolar extinction coefficient of 6.22 was used to calculate the rate of NADH disappearance.

Spectrophotometric measurement of protein disulfide-thiol interchange activity - Protein disulfide-thiol interchange interchange activity was monitored spectrophotometrically at 340 nm by following the cleavage of 2,2′ dithiopyridine.



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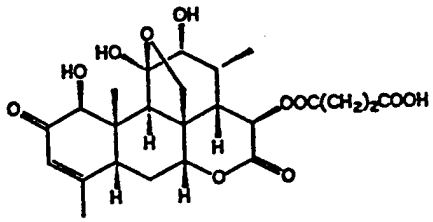

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Glaucarubolone-PEG conjugation - Glaucarubolone (1) isolated from the roots of *Castela polyandra* was provided by Dr. Paul Grieco, Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT (see below). A C(15) succinylated derivative of glaucarubolone 2 was coupled to amino polyethyleneglycol (ave MW 5000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

RESULTS AND DISCUSSION

In order to support a significant number of animal tests, 500 mg of glaucarubolone was prepared for the project by Dr. Paul A. Grieco, Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana by extraction from the root bark of the desert shrub *Castela polyandra*. In addition another 500 mg of derivatized glaucarubolone was prepared for linking the amino

polyethyleneglycol to form the glaucarubolone-polyethyleneglycol (glaucarubolone-PEG) conjugate.

For stereospecific conjugation via the $C_{(15)}$ hydroxyl group, we prepared II (Fig. 1) using a procedure that differentiated the $C_{(1)}$, $C_{(12)}$ and $C_{(15)}$ hydroxyl groups in glaucarubolone. Exposure of glaucarubolone to TMSOTf in pyridine containing triethylamine (0° C, RT, 1 h) gave rise (75% yield) to tris-trimethylsilylated material III (Fig. 1) in which the $C_{(12)}$ hydroxyl was exposed. Exposure of III to tetrabutylammonium fluoride in THF cleaved exclusively in excellent yield the $C_{(15)}$ OTMS ether. Treatment of IV (Fig. 1) with succinic anhydride in methylene chloride containing trimethylamine and 4-dimethylaminopyridine gave rise (80%) after treatment with HF/CH_3CN to II (Fig. 1) which was then conjugated with amino polyethyleneglycol. Note that the $C_{(12)}$ hydroxyl was sufficiently hindered to allow stereospecific acylation of the $C_{(15)}$ hydroxyl giving rise exclusively to II (Fig. 1). The derivatized glaucarubolone (II, Fig. 1) was coupled to amino polyethyleneglycol (Ave MW 5,000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

A series of specific tests were carried out to evaluate the effect of both the quassinoid glaucarubolone, its conjugate with polyethyleneglycol (PEG) and the vanilloid, vanillylamine on different metastatic parameters in laboratory models. These studies were carried out in collaboration with the laboratory of Prof. Marc Mareel, University Hospital, Ghent, Belgium. To measure the epithelial Ca^{2+}-dependent cell-cell adhesion mediated by E-cadherin when linked to the actin cytoskeleton via various catenins, the slow aggregation assay was carried out (Table 1) comparing two human breast cancer cell lines (MCF-7/AZ and MCF-7/6) and a human colonic carcinoma (HCT-8/R1). At the concentrations tested, none of the drugs had any effect on cell aggregation such that inhibitory effects were not directed to involvement of the E-cadherine-α-catenin system.

Collagen invasion assays, in contrast, did reveal activity (Tables 2 and 3). Here, type I collagen was mixed with culture medium to achieve a final concentration of 0.09% collagen. The ability of cells to invade into the collagen was estimated microscopically after 24 and 48 h. Results are expressed as percent of cells invading. The DHB-FIB is an invasive cell line whereas the MCF-7/AZ is a non-invasive breast cancer cell line.

In the first experimental series with the collagen invasion assay (Table 2), invasion with the invasive DHD-FIB cell line was reduced from 8.4% to 2.0% by a 10^{-8} M concentration of the glaucarubolone-PEG conjugate. The conjugate was at least twice as effective as free glaucarubolone. Capsaicin at 1 μM also inhibited in the collagen invasion assay.

In the second experimental series with the collagen invasion assay (Table 3), the glaucarubolone-PEG conjugate was most effective at a concentration of 10^{-10} M and equivalent at that concentration to 1 μM capsaicin and more effective than 0.01 to 1 μM of the free glaucarubolone. The concentration dependence in this assay was unusual and similar anomalies were observed in experiments with isolated plasma membrane vesicles in the inhibition of NADH oxidase (Fig. 2).

In the chick heart invasion assay (Table 4), cells were confronted with fragments of embryonic chick heart and invasion was scored on serial histological sections. With the PHF/MO4 cells on day 4, there was no effect of any of the compounds tested on invasion of the chick heart fragments. However, with the PHF/Bowes Melanoma on day 4 there was a response to the glaucarubolone-PEG conjugate not given by free glaucarubolone (Table 4).

A problem with glaucarubolone as a cancer therapeutic agent has been its rather low margin of safety. This is illustrated in Table 5 where the EC_{50} for free glaucarubolone for the human mammary epithelial cell line MCF10A is only one order of magnitude greater than that for the human mammary carcinoma cell line BT-20. The murine 4T1 breast cancer cell line is as resistant to free glaucarubolone as is the mammary epithelia cell line MCF10A. However, when tested with the glaucarubolone-PEG conjugate, the EC_{50} is shifted by about 1 order of magnitude to lower drug concentrations.

Both the vanilloid vanillylamine and the quassinoid glaucarubolone were tested in the BALB/c mice carrying subcutaneous experimental tumors derived from the 4T1 cell line. The dose limiting toxicity of vanillylamine had been determined previously.

Vanillylamine given at a dose of 1 mg/mouse per injection beginning on alternate days after palpable tumor masses were discernable for a total of 6 injections was without effect either on tumor growth or lung metastases (Table 6). However, when combined with 0.2 mg ascorbate/animal/injection, a reduction both of tumor mass and numbers of metastases was observed. Ascorbate alone at 0.2 mg/animal/injection was without effect. For vanillylamine to be active in restricting tumor growth and metastases, the target protein must be oxidized. This is effected in the experimental system with either ascorbate (Table 6) or t-butylhydroperoxide (e.g., Table 3).

With free glaucarubolone, the two highest doses of 5 and 10 mg/animal/injection were toxic. Even at the near maximum tolerated dose of 2.5 mg/animal/injection, no tumor response was seen either from the standpoint of inhibition of growth of the primary tumor of inhibition of metastases to the lungs of the animals bearing tumors (Table 7).

In contrast to free glaucarubolone, the conjugate of glaucarubolone with PEG was not toxic at 5 mg/animal/injection (Table 8). At this concentration, the glaucarubolone-PEG conjugate was cytostatic to the growth of the murine mammary carcinoma cells in the mice (Fig. 3) whereas at 2.5 mg/animal/injection, the tumors grew to approximately the same size as controls.

Despite the cytostatic observations with the glaucarubolone-PEG conjugate at day 5 following the beginning of treatment, there was no significant slowing of tumor growth or of metastasis at the end of the experiment (Table 8). Higher concentrations of the glaucarubolone-PEG conjugate could not be tested due to problems of solubility.

RECOMMENDATIONS

Drug conjugation emerges as a viable strategy to reduce toxicity of anti-cancer drugs targeted to the cancer specific NADH oxidase without loss of antitumor activity. With the glaucarubolone-PEG conjugate, problems with solubility and tissue penetration have been encountered. New glaucarubolone conjugates with lower molecular weight amino polyethylene glycols will be designed, synthesized and tested. Also to be designed, and tested will be additional, potentially more effective drugs based on the vanilloid series of tNOX inhibitors.

CONCLUSIONS

Both the quassinoid glaucarubolone and the vanilloids vanillylamine and capsaicin, inhibitors of the cell-surface, unregulated and drug-responsive NADH oxidase over expressed in cancer cells (tNOX) show antimetastatic activity in breast cancer models. Use of both capsaicin and free glaucarubolone in animal experiments is compromised by high toxicity and a low therapeutic ratio. However, with the conjugated glaucarubolone and with vanillylamine, dose-limiting toxicities have not been encountered. Drug conjugation is clearly a viable strategy to reduce toxicity without loss of antitumor activity with the tNOX inhibitors. The conjugate will be redesigned in the 02 year of the project to attempt to overcome these problems and the new conjugates will be tested. More effective NOX inhibitors related to the vanilloids are being sought and tested. One, a catechin, appears to be at least two orders more effective than glaucarubolone and is currently being prepared for evaluation in the mouse model both as the free drug and as a conjugate.

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Thus far, one publication has resulted:

Morré, D. J., Grieco, P. A. and Morré, D. M.. In Press. Mode of action of the anticancer quassinoids-inhibition of the plasma membrane NADH oxidase. Life Sciences.

TABLE 1. Slow aggregation assay with MCF-7/6 cells.

Drug	MCF-7/AZ	MCF-7/6	HCT-8/R1
Untreated	+	±	-
Capsaicin 1 μM	+	±	-
Vanillylamine 10 μM + Ascorbic acid 10 μM	+	±	-
Glaucarubolone 10^{-8} M	+	±	-
Glaucarubolone-PEG conjugate 10^{-8} M	+	±	-

The MCF-7/6 cells formed aggregates with irregular borders. There was more compaction and larger aggregates than usual.

TABLE 2. Collagen invasion assays.

Cells	Treatment	Percent invasion
DHD-FIB	None	8.4
DHD-FIB	1 μM Capsaicin	5.7
DHD-FIB	10 μM Vanillylamine and 10 μM Ascorbate	7.4
DHD-FIB	10^{-8} Glaucarubolone	4.7
DHD-FIB	10^{-8} Glaucarubolone-PEG conjugate	2.0
MCF-7/AZ (noninvasive)	None	0.0

TABLE 3. Collagen invasion assays continued.

Cells	Treatment	Percent invasion
DHD-FIB	None	18.4
DHD-FIB	10^{-6} Glaucarubolone	8.3
DHD-FIB	10^{-7} Glaucarubolone	5.3
DHD-FIB	10^{-8} Glaucarubolone	5.2
DHD-FIB	10^{-8} Glaucarubolone-PEG conjugate	7.4
DHD-FIB	10^{-9} Glaucarubolone-PEG conjugate	3.8
DHD-FIB	10^{-10} Glaucarubolone-PEG conjugate	2.8
DHD-FIB	1 μM Capsaicin	2.9
DHD-FIB	10 μM Capsaicin + 10 μM t-Butylhydroperoxide	6.0
DHD-FIB	10 μM t-Butylhydroperoxide	3.7
MCF-7/AZ	None	0.0

TABLE 4. Chicken heart invasion assay.

Confrontation	Grade of Invasion					
	0	I	II	III⁻	III	IV
PHF/MO4: d_4						3
+ Capsaicin						3
+ Vanillylamine + Ascorbic acid						3
+ Glaucarubolone-PEG conjugate						3
PHF/Bowes Melanoma: d_{a4}	1				2	
+ Capsaicin	1		1		1	
+ Vanillylamine + Ascorbic acid						3
+ Glaucarubolone						3
+ Glaucarubolone-PEG conjugate			2		1	

Concentrations as in Tables 1-3.

TABLE 5. EC_{50} in cell culture of glaucarubolone and the glaucarubolone-PEG conjugate.

Cell line	Glaucarubolone	Glaucarubolone-PEG conjugate
HeLa[1]	0.5 μM	1 μM
MCF10A[2]	1 μM	-
BT-20[3]	0.1 μM	-
4T1[4]	> 1 μM	< 1 μM

[1]Human cervical carcinoma
[2]Mammary epithelia (non-cancer)
[3]Human mammary adenocarcinoma
[4]Mouse mammary adenocarcinoma

TABLE 6. Effect of the vanilloid, vanillylamine, with and without ascorbic acid on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice.

Compound	Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	-	0.6 ± 0.2	2.8 ± 3
Vanillylamine	1 mg	0.64 ± 0.15	4 ± 1.6
Ascorbate	0.2 mg	0.6 ± 0.2	3.4 ± 1.5
Vanillylamine + Ascorbate	1 mg + 0.2 mg	0.48 ± 0.2	1.8 ± 1.3

Cells from monolayer culture were suspended in Hank's buffered salt solution and 1 x 10^6 cells were injected into 5 mice/treatment in the subscapular region in a volume of 0.1 ml. Primary tumors were measured twice a week in 2 perpendicular dimensions using a vernier caliper. Tumor mass in cm^3 was calculated by the formula $a \times b^2/2$, where b is the smaller and a is the larger of the two dimensions.

Anti-metastatic drugs were administered in the dose/injection indicated intratumoral in 0.1 ml total volume on alternate days beginning after palpable tumor masses were discernible (approximately 7 days post implantation) for a total of 6 injections. Appropriate solvent and sham-injected controls were included. At 15 days post tumor implantation, mice were sacrificed and major organs (e.g., lung, liver, lymph nodes) were examined for evidence of metastases.

TABLE 7. Effect of glaucarubolone on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice. Experimental details as for Table 6.

Glaucarubolone, Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	1.1 ± 0.3	2 ± 2
1 mg	0.6[5]	11
2.5 mg	1.0 ± 0.5	4 ± 2
5 mg	Dead	
10 mg	Dead	

TABLE 8. Effect of glaucarubolone-PEG conjugate on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice. Experimental details as in Table 6.

Glaucarubolone-PEG conjugate Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	3.3 ± 1.2	4 ± 0
1 mg	2.4	5
2.5 mg	2.9 ± 0.7	5 ± 2
5 mg	2.6	3

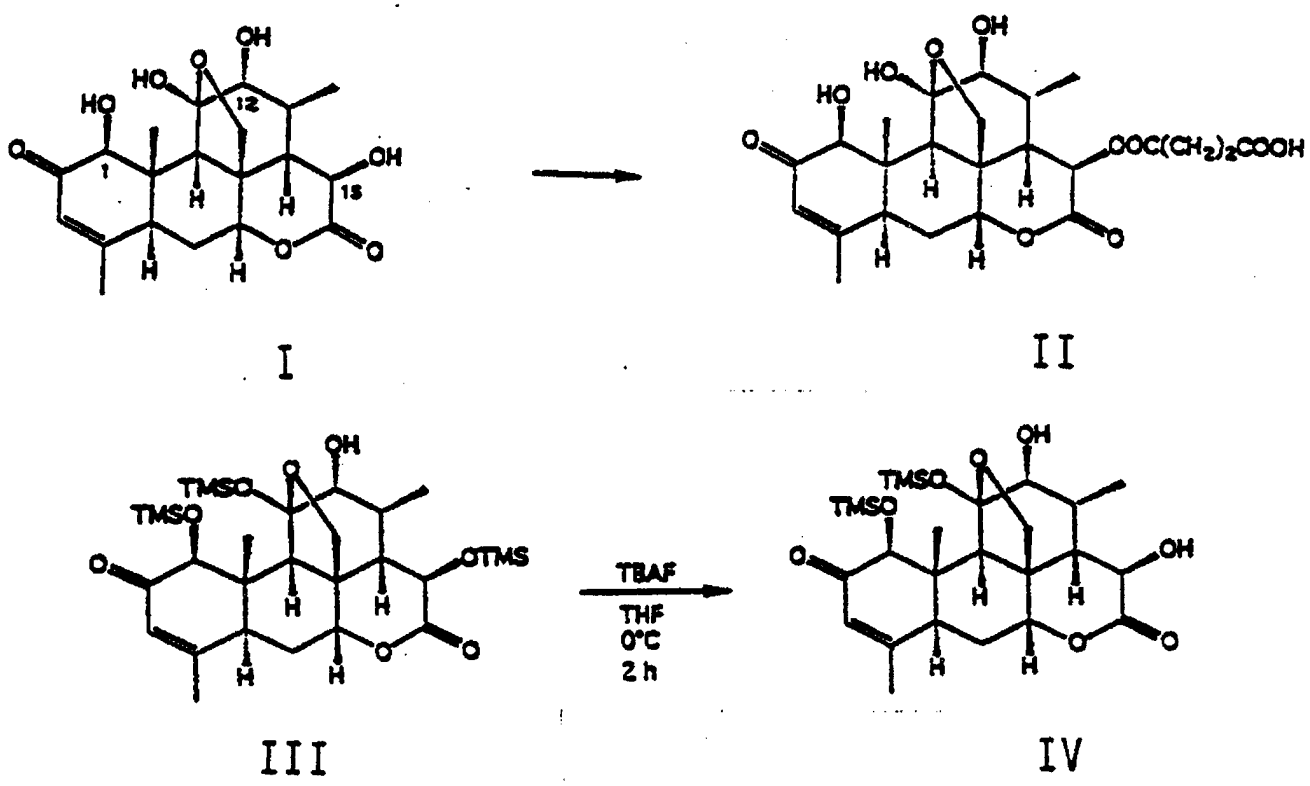

Fig. 1

Structure of glaucarubolone (I) and intermediates (III, IV) in the formation of the derivitized glaucarubolone (II) for coupling to amino polyethyleneglycol.

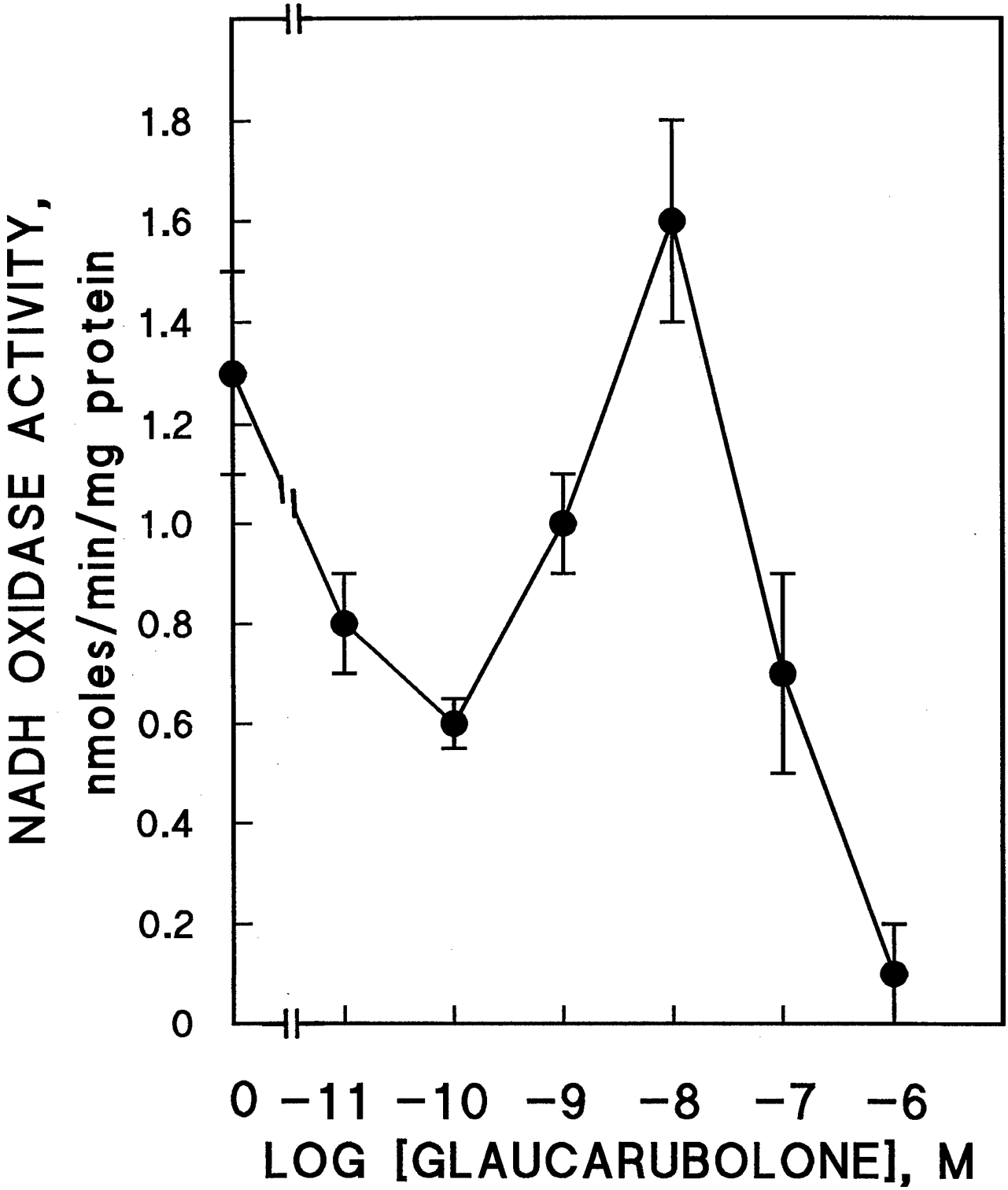

Fig. 2

Dose response of the NADH oxidase activities of plasma membrane vesicles from HeLa cells to glaucarubolone. Values are averages ± standard deviations.

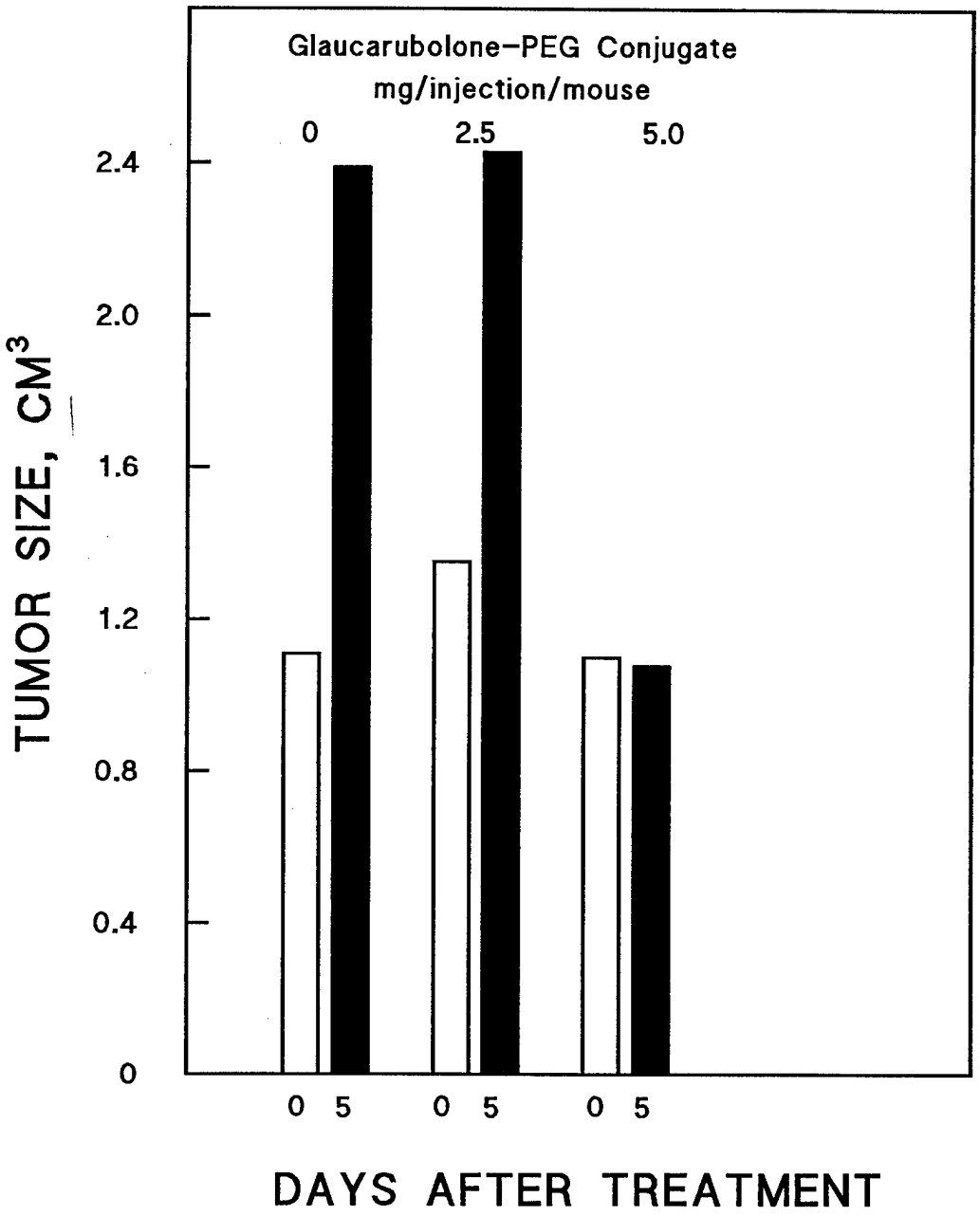

Fig. 3

Tumor mass, calculated in cm^3, by the formula $a \times b^2/2$ where b is the smaller and a is the larger of the two dimensions comparing tumors after 0 and 5 days of treatment for mice receiving no treatment (0) and 2.5 or 5 mg of glaucarubolone-PEG conjugate per injection per mouse.